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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The purpose of this study was to develop an <u>in vitro</u> model stimulating carious dentin overlying chronically inflamed pulpal tissue for the subsequent evaluation of drug penetration into pulps <u>in vivo</u> . Dentin disks 1.03 + 0.01mm thick and 10mm in diameter, sectioned from non-carious human molars, were inserted in plastic split chambers designed as models of human teeth. To produce affected dentin, one half mL of each of four different decalcifying agents tested was placed on the occlusal side of the chamber in contact with the dentin disk and sealed. One mL of pH 7.6 phosphate buffered saline (PBS) was placed on the opposite (pulpal) side. This treatment was designed to produce moderate dentin demineralization with a gradual shift in pulpal pH from 7.6 to an acidic state of 6.8 similar to that produced <u>in vivo</u> by slow penetration of bacterial acids. The pH of the PBS was determined at 24 and 48 hours. Of the 4 agents tested, 2.5% citric acid and 6% lactic acid both reduced the pulpal pH from 7.6 to 6.8 +/- 0.1. SEM photomicrographs of the acid-exposed dentin, compared to <u>in vivo</u> bacterially produced dental caries, appeared qualitatively similar with 6% lactic acid most closely resembling the natural caries. In the second phase of this study, drug penetration through					
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demineralized dentin was evaluated in the model in the presence of additional physiological variables. Specifically, pulpal fluid contains protein, which increases during inflammation as pH drops. Therefore 6.5% serum albumin was also added to the elution buffer in the pulpal chambers in the presence of both normal and inflamed pH. Each variable; condition of dentin, pH, and presence of protein, was found to directly and significantly alter drug movement to a degree and extent dependent on the combinations present. As each variable tested also brings the model closer to physiologic reality, it is concluded that each be present when the model is used for in vitro studies directed at predicting and optimizing movement of drug thru carious dentin into inflamed pulps in vivo.

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UNITED STATES ARMY INSTITUTE OF DENTAL RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D.C. 20307-5300

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Department of Oral Biology
Faculty of Dentistry
University of Manitoba
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Enclosure
Manuscript (triplicate)

**A Model System for Predicting Drug Penetration
Thru Decayed Dentin Into Inflamed Pulp**

G.F. SENG AND M.F. GROWER

**US ARMY INSTITUTE OF DENTAL RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, DC 20307-5300**



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ABSTRACT

The purpose of this study was to develop an in vitro model simulating carious dentin overlying chronically inflamed pulpal tissue for the subsequent evaluation of drug penetration into pulps in vivo. Dentin disks 1.03 ± 0.01 mm thick and 100 mm in diameter, sectioned from non-carious human molars, were inserted in plastic split chambers designed as models of human teeth. To produce affected dentin, one half mL of each of four different decalcifying agents tested was placed on the occlusal side of the chamber in contact with the dentin disk and sealed. One mL of pH 7.6 phosphate buffered saline (PBS) was placed on the opposite (pulpal) side. This treatment was designed to produce moderate dentin demineralization with a gradual shift in pulpal pH from 7.6 to an acidic state of 6.8 similar to that produced in vivo by slow penetration of bacterial acids. The pH of the PBS was determined at 24 and 48 hours. Of the 4 agents tested, 2.5% citric acid and 6% lactic acid both reduced the pulpal pH from 7.6 to 6.8 ± 0.1 . SEM photomicrographs of the acid-exposed dentin, compared to in vivo bacterially produced dental caries, appeared qualitatively similar with 6% lactic acid most closely resembling the natural caries. In the second phase of this study, drug penetration through demineralized dentin was evaluated in the model in the presence of additional physiological variables. Specifically, pulpal fluid contains protein, which increases during inflammation as pH drops. Therefore 6.5% serum albumin was also added to the elution buffer in the pulpal chambers in the presence of both normal and inflamed pH. Each variable; condition of dentin, pH, and presence of protein, was found to directly and significantly alter drug movement to a degree and extent dependent on the combinations present. As each variable tested also brings the model closer to physiologic reality, it is concluded that each be present when the model is used for in vitro studies directed at predicting and optimizing movement of drug thru carious dentin into inflamed pulps in vivo.

INTRODUCTION

Patients seeking emergency treatment for non-traumatic pain of odontogenic origin are frequently manifesting the symptoms of "acute" pulpitis, and caries is the major cause of pulpitis (Trowbridge, 1985). Such acute painful pulpitis which is the inevitable sequelae of untreated dental caries, if untreated, will progress to a chronic pulpal inflammation or pulpal death (Ingle et al., 1976; Simon, 1980; Seltzer and Bender, 1984). In decayed human teeth bacterially produced acids demineralize dentinal tubules producing "affected dentin" (Massler, 1972). The acids and other metabolic by-products then transverse the tubules into the pulp and produce tissue damage and inflammation (pulpitis) (MacGregor, 1961; Massler and Pawlak, 1977). To approximate the natural state, in vitro studies directed at evaluating the penetration of therapeutic pharmacological agents through dentin into such "inflamed" pulps should utilize dentin similar in structure/composition to demineralized carious dentin produced in vivo. Also, the inflamed pulpal tissue/fluid substitute used in vitro should also approximate the in vivo state, as multiple pharmacokinetic parameters in inflamed tissue interact to, in turn, alter drug pharmacodynamics.

A host of innovative and elegant studies of dentin and dentin permeability have been conducted by the highly esteemed group at Georgia led by Pashley, Outhwaite, Reeder, et al. (see footnote).

OUTHWAITE, et al., 1974, 1976; MERCHANT, et al., 1977; MICHELICH, et al., 1978; REEDER, et al., 1978; PASHLEY, et al., 1978, a,b,c; 1980; 1981 a,b; 1982; 1983 a,b,c,d; 1984 a,b,c,d; 1985; 1987 a,b; 1989; PASHLEY, 1979; 1984; 1985; 1986; 1989; FOGEL, et al., 1988

Studies by others have been directed at time release and diffusion of pharmacologic agents through dentin (Hume & Kenney, 1981; Hume, 1984; Lindemann et al., 1985). In none of the studies cited, however, was dentin chronically exposed to acid for sufficient time such that the acid could penetrate and demineralize the entire thickness of the dentin to the depth of the "pulp." For example, Michelich et al. (1987) etched the pulpal side of dentin discs for 1 minute with 50% citric acid. Pashley et al. (1978) etched both sides of discs with 50% citric for 2 minutes. However, these and similar 2 minutes exposures of 1 mm thick discs to 50% citric have been shown to only remove surface debris and peritubular dentin to a depth of 20 um (Brannstrom and Johnson, 1974; Pashley et al., 1978) leaving 80% of the tubule length virgin (60% if both sides are etched). Such exposure may also leave remnants of, and residue from, odontoblastic processes in the tubules as these have been shown extending at least 35% of the distance from the pulp to the D.E.J. (Brannstrom and Garberoglio, 1972; Tsatsas and Frank, 1972; and Holland, 1975, 76).

A second significant issue to be addressed when utilizing split chamber devices (Outhwaite et al., 1974), as human teeth models, is that of the pulpal substitutes similarity to actual inflamed endodontic tissue. For example, there are anticipated and obvious pH changes in inflamed tissue (Brune and Graf, 1968), as well as the subsequent extravasation of plasma protein into the extracellular fluid of inflamed pulpal tissue (Mjor, 1985). That such proteins can subsequently penetrate dentin (Pashley et al., 1982; Pashley et al., 1983_a) and then effect dentin permeability has been demonstrated in vitro (Pashley et al., 1983_b, 1984_a) and in vivo (Pashley et al.,

1984_{a,b}). In addition, plasma proteins have a pronounced effect on the pharmacokinetic activity of most drugs due to changes in osmotic balance, as well as the varying protein binding affinities of different drugs, and the "bumping" phenomenon (Goodman and Gilman, 1980).

Finally, it should be noted that many of the previous studies utilized hydraulic conductance driven by the force of gravity imparted by a 240 cm column of PBS buffer solution which created a constant hydrostatic pressure forcing solute through dentin, (Merchant et al., 1977). While appropriate for the issues addressed in the works cited, this approach was at slight variance with the physiologic conditions that the model being developed in this study attempted to address.

The studies in this paper were directed at developing an in vitro model for reproducibly creating demineralized dentin simulating carious tooth dentin, as well as at closely approximating the physiologic conditions in inflamed pulps. The techniques were also developed so that they could subsequently be utilized in vivo in animals to consistently create demineralized dentin and concomitant pulpal inflammation without pulpal death.

METHODS

Freshly extracted non-carious human molar teeth were immediately submerged in isotonic phosphate buffered saline (PBS) solution, pH 7.6 containing 0.1% sodium azide as an antimicrobial, for storage prior to use. It has been previously reported that post-extraction storage time does not affect dentin permeability (Outhwaite et al., 1976; Reeder et al., 1978). Dentin sections were obtained from the occlusal surfaces of individual teeth which were attached with epoxy resin to a mounting jig from an Isomet®

slow-speed diamond jeweler's saw. Discs 1.03 ± 0.01 mm (\pm STD error of mean, $N = 61$) thick and approximately 100 mm in diameter were sectioned from the crown of the tooth moving from pulpal to occlusal with the Isomet saw. Discs up to and including that containing the last visible remnant of pulp horn (and those showing any evidence of natural decay) were discarded and the next most coronal 1 mm disc was used in these experiments. Cut discs were stored in the PBS solution prior to use. Each disc to be used was inserted into a plastic split chamber similar to that described by Reeder et al. (1978) with significant modifications by Seng and Grower (1989) (see Fig. 1). The chambers were designed to structurally and qualitatively resemble human teeth. The dentin discs were sealed into the chambers with "O" rings in the proper occlusal-pulpal orientation. In the initial screening experiments, 1.0 mL of 0.002 M PBS (representing the pulp in its entirety) was placed into the pulpal (bottom) section of the chamber in direct contact with the pulpal side of the dentin. The occlusal (top) section of the chamber over the occlusal dentin was filled with 0.5 mL of selected concentrations of lactic (5 - 10%), citric (2.5 - 10%), phosphoric (2 - 10%), or acetic (4 - 10%) acids to determine which concentration(s) of which acid(s) would most reproducibly reduce pH in the "pulpal tissue/fluid" from the neutral pH of 7.6 to a moderately acidic condition of 6.7 ± 0.3 (\pm SEM). pH changes in the PBS solution representing pulpal tissue at 25°C were then determined at 24 and 48 hours utilizing a Beckman 701 pH meter and a Fisher micro-electrode.

In subsequent experiments, the PBS buffer concentration was increased to the more physiologic concentration of 0.01 M and the temperature of incubation of acid and buffer increased to 37°C to more closely approximate in vivo

conditions at the pulpal-dentin interface. In addition, when drug penetration studies were conducted, physiologic concentrations (Scientific Tables, 1971) of protein (6.5% albumin) were added to the pulpal buffer to simulate the extravasated fluid and protein found in inflamed pulpal tissue.

Acid treated discs were examined with an Amray 1645 scanning electron microscope and compared to similar sections taken from natural caries (affected dentin). In addition, demineralized discs placed in the test chambers were exposed to a 0.015% Coomassie Brilliant Blue dye to determine the degree to which dye would transverse the artificially decayed (demineralized) dentin as compared to non-demineralized dentin.

Measurement of drug penetration through the dentin disc was accomplished using the non-steroidal anti-inflammatory agent indomethacin, in varying concentrations (3 - 10 ug/mL), which was combined with either the ^3H or ^{14}C radioactively labeled forms and incorporated into several time release vehicles. The vehicles utilized, as well as the labeling isotopes, are presented in Table 1.

As dentin tubules can vary greatly in both number and diameter, dialysis tubing (Spectra/POR® Membrane, 6000-8000 molecular weight cutoff) was substituted for dentin in some studies to evaluate drug movement thru a uniform "fixed"-pore-size system. The tubing was inserted into the chambers in the place of dentin discs and exposed to vehicles containing indomethacin. This system was used to evaluate the effects on drug movement that would be exerted by the presence of proteins and the acid pH that would be encountered in an inflamed pulp.

Finally, the various drug vehicle combinations were applied to decalcified and undecalcified dentin discs mounted in the chambers against pulpal fluid with and without protein, and at physiologic and inflammatory (acidic) pH.

Appearance of radioactively labeled drug in the pulpal fluids following the passage through dialysis tubing and dentin was determined and quantitated by liquid scintillation spectroscopy.

RESULTS

Figure 2 shows the results of initial experiments in which several of the various acid concentrations utilized at room temperature were tested. Both 6% lactic and 2.5% citric acid at 25°C consistently and reproducibly lowered the pulpal pH of 0.002 M PBS to the desired 6.7 ± 0.3 (\pm SEM) range over 48 hours. Graded concentrations (2% - 10%) of the other acids produced more erratic and less consistent results.

In addition, a light white precipitate was observed on the occlusal portions of the discs treated with citric, acetic, and phosphoric acids. A similar heavier precipitate was observed on the pulpal surface of the discs and in the pulpal PBS. The degree of precipitate appeared directly proportional to the concentration of the acids. No such precipitate was observed with lactic acid. Electron photomicrographs indicated the crystals to be needle-like in structure.

Further studies were done to develop a more physiologic system by determining the effects of changes in buffer concentration and temperature on the pH changes observed in the model system. Figure 3 shows the results of these studies. It was determined that at 37°C, 6% lactic acid was effective in

causing a gradual pH shift from an initial pH of 7.6 to 7.1 ± 0.2 (\pm SEM, $n = 15$) at 24 hr and to 6.7 ± 0.3 (\pm SEM) at 48 hr when the physiologic concentration of 0.01 M PBS buffer was used in the incubation chamber.

When electron photomicrographs of discs of bacterially affected, carious dentin produced in vivo were compared to discs exposed in vitro to the different acids, only 6% lactic acid produced dentinal tubule demineralization which appeared similar to caries (see Figs. 4a,b,c,d).

Dentin discs, which were demineralized with 6% lactic acid for 48 hours and then exposed to low molecular weight Coomassie Brilliant Blue dye in the model system, allowed detectable amounts of dye to traverse the dentin over 24 hours (Table 2). Discs not treated with acid allowed no penetration.

Initial pilot studies indicated that if protein was not included in the pulpal buffer solution, indomethacin penetration, though minimal, especially at 24 hours, was greater through the undecalcified than the decalcified discs (Table 3). This occurred even though tubule diameter was larger in decalcified discs (Figs. 4b,d) than the undecalcified discs (Fig. 5). This may have resulted from the lack of protein in the pulpal buffer creating a non-physiologic state due to a combination of the ion exchange properties of the dentin tubules, (Budz, et al., 1988; Hoppenbrowers and Driessens, 1988), the osmotic effect and hydrophilic nature of the delivery vehicles, and/or the poor water solubility of the indomethacin as well as its protein binding activity (Yeh, 1985).

In any case as the pulpal fluid, which penetrates dentinal tubules in vivo contains protein (Von Kreudenstein 1955; Pashley et al., 1982; Pashley et al., 1983), serum albumin was added in physiologic concentrations (Scientific Tables, 1979) to the pulpal buffer used in further experiments.

To evaluate drug movement in a controlled environment with the only variables being the presence or absence of protein in the buffer and the pH difference (7.6 vs. 6.8), dialysis tubing was utilized in the split chamber instead of dentin in the next series of experiments. Table 4 shows the results obtained using ^{14}C labeled indomethacin in a polyethylene-propylene glycol vehicle at pH 7.6 or 6.8 with and without albumin in the incubation buffer. With albumin present, approximately 20 ug of indomethacin passed into each mL of pH 7.6 buffer on the pulpal side. Without albumin, a dramatic and significant ($P < 0.02$) drop to only 10% of the values with albumin present occurred both at 24 and 48 hours. When pulpal side pH was lowered to 6.8, the decrease in drug movement was limited to approximately 50% (9.8 ± 3.3) at 24 hours, which was reduced again by half (4.5 ± 2.6) at 48 hours. Even these values, however, represent a threefold greater drug movement at 24 hours with albumin than without (3.0 ± 1.5), and despite a decrease to 4.5 ± 2.6 ug/mL at 48 hours, it remains 50% greater at that point when compared to the buffer without protein.

Having demonstrated the effects of protein and pH in an inert, fixed diameter system, acid treated (decalcified) and untreated (undecalcified) dentin discs were again utilized. The discs were sealed into the chambers and exposed to ^{14}C labeled indomethacin in a polyethylene-propylene glycol vehicle on the occlusal side and pulpal side buffer containing 6.5% serum albumin. Results shown in Table 5 reveal a 50% greater accumulation of drug on the pulpal side of the decalcified discs when compared to the undecalcified regardless of pH. The difference between the samples incubated at pH 6.8 was significant at $P < 0.05$ to $P < 0.005$. There were no statistically

significant differences between the drug levels observed at 24 and 48 hours between any of the groups in this experiment. While the 50% decrease in drug movement thru undecalcified discs may be attributable to the presence of the protein or the inflammatory pH, it is more likely a result of the combination of tubule diameter and tubule contents with these two variables.

Table 6 shows a comparison of drug movement through acid treated (decalcified) vs. untreated discs, and the effects of pulpal side pH changes, all with protein present in the system. Indomethacin in 2 different vehicles labeled with [14 C] indomethacin was placed on the occlusal side of undecalcified and decalcified dentin discs, pulpal side pH was either 7.6 (physiologic) or 6.8 (inflamed).

At the inflammatory pH of 6.8, acid treated dentin permitted 2 - 5 times more drug through dentin at 24 hours than undecalcified dentin regardless of vehicle. At 48 hours this decreased to less than 2 - 3 fold indicating movement through decalcified discs reached an equilibrium and stabilized while movement through undecalcified discs continued to increase by 50% with both Inteban and glycerin vehicles. With acid treated discs, the pH of pulpal buffer exerted only minor or no effects at 24 hours while a detectable difference between the two pHs appeared at 48 hours; 25% less drug appeared on the pulpal side at pH 7.6 after 48 hours compared to the concentrations found in the pulpal PBS at pH 6.8.. In addition, the delivery vehicle Inteban released more drug through dentin by a ratio that varied from as low as 3:1 up to as high as 10:1 when compared to the glycerin based delivery vehicle.

The increased indomethacin penetration into pulpal buffer at 24 hours, seen earlier in the undecalcified sections when albumin was not present, did

not repeat under the altered and more physiologic parameters of the model as described now with albumin present.

When the 2 different vehicles releasing drug through decalcified dentin into the physiologic buffer with a pH of 7.6 are compared (Table 6), the amount of drug on the pulpal side appeared to decrease between 24 and 48 hours, which may indicate a reverse movement of the drug back into the drug reservoir. It may also be due, at least in part, to either a dilution of drug in the reservoir since samples were incubated for 24 hours previous to 2nd incubation and/or filling of the tubules with delivery agent. When the pH on the pulpal side of the decalcified discs was reduced to inflammatory levels, the apparent reverse movement ceased. This could be a reflection of a form of ion trapping if indeed the proteins were occluding the tubules to a degree creating a semi-permeable effect. There also may have been some precipitation of this insoluble drug as a result of the more acidic environment. Finally, with the non-decayed discs, drug continued to move thru the dentin up to 48 hours showing a significant increase over the initial 24 hour period.

DISCUSSION

The purposes of these studies were twofold: The more immediate goal was to produce an in vitro model of decayed dentin in which to evaluate drug conductance through dentinal tubules; the long range goal was to establish a system for creating both quantifiable and reproducible demineralized dentin (decay) and chronic pulpal inflammation (pulpitis) to be used in in vivo animal studies. 6% lactic acid produced pH changes within both the pre-determined pH range (6.7 ± 0.3) and time period (48 hours). Based on tissue-

culture studies, this pH range was chosen in anticipation that the acid, after traversing and demineralizing dentin, would in subsequent animal experiments produce an inflammatory response in vivo in the pulp without causing death of that pulp, (Seng and Bayer, 1986). The 6.7 ± 0.3 range is similar to that observed in the exudate in moderate inflammatory condition (Brune and Graf, 1968), and the acid concentration producing the desired pH change within 48 hours, approximates the time Lervik and Mjor (1977) found for human decay to cause pulpal inflammation when placed in preparations in animal teeth.

The time frame of 48 hours was chosen to ostensibly produce a slowly developing "chronic" (cellularly defined) inflammatory response, rather than an acute response because most pulpitis due to caries are chronic in nature as opposed to acute pulpitis caused by trauma. Acute inflammatory cells, the polymorphonuclear leukocytes (PMN) are gradually replaced in inflamed tissues by lymphocytes beginning around 24 hours and by 48 hours these are the predominate cells and the process is then considered chronic (Robbins et al., 1984).

The chamber volumes were selected based on an effort to maintain proportionate in vivo anatomic ratios. For example, total pulp volume in young molar teeth approximates 0.5 mL (Cohen and Burns, 1980) and indirect pulp capping material volume is reasonably estimated at 250 ul. Both these volumes were doubled in the chamber design to maintain proportionate sizes while facilitating laboratory procedures. Inflow and outflow portals were added to the pulpal side, so that when attached to a fraction collector, pulpal blood flows ranging from zero (total ischemia) to a normal pulpal flow

of 50 mL/100 grams/min (Meyer, 1980 and Kim et al., 1980) could be reproduced and pulpal drug concentrations measured at the different flow rates. In these initial studies, however, the portals were sealed and a static system was used.

Again, the selected pH drop from 7.6 to 6.7 ± 0.3 was of a magnitude anticipated to subsequently produce demineralized dentin and produce chronic pulpal inflammation in vivo in the teeth of experimental animals without causing pulpal death.

When electron photographs of discs cut through actual carious dentin from decayed teeth were visually and subjectively compared to discs exposed to the various acids, 6% lactic acid appeared to most closely approximate the in vivo decay as far as tubule aperture diameter, lack of debris and absence of constrictions. While efforts directed at mathematically quantitating or comparing the differences were not considered of significant importance to the initial development of the basic model, it could be readily done in future studies.

Dentin discs were exposed to 0.0015% Coomassie Brilliant Blue dye after demineralization to determine if substances with a larger molecular size than any drug that would be tested would pass through the dentin into the "pulp". Calculations of bond lengths in the indomethacin molecule indicate the molecule diameter to be from 13.8 to 14.5 angstroms, depending on the orientation of the molecule (Rebert, 1989). The largest diameter of 0.0015% Coomassie Brilliant Blue dye G-250 is 18.5\AA (Rebert, N. 1989). Dentinal tubule diameter has been determined to vary from 0.5 - 0.9 μm at the dento-enamel junction to 2.3 μm at the pulpal interface (Tronstadt 1973; Garberoglio and

Brannstrom, 1976). Dye readily passed through the tubules in the acid treated dentin, but not through the untreated dentin over a 24 hour period. These findings are in agreement with the work of Greenhill and Pashley, (1981) who showed that, in dynamic systems where positive pressures were applied to dentin discs along with fluid efflux, the fluid flow of plain Krebs-Ringer's phosphate (0.01 M phosphate, pH 7.4) buffer through dentin tubules varied with the 4th power of the radius of the tubules. These results also are in consonance with findings of Michelich et al. (1978) and Pashley (1985) indicating that the functional radius of the tubules in the dentin discs range from 5 - 40% of the anatomic (SEM) radii. This is due, as they point out, to the fact that the tubules actually contain fibrillar structures, odontoblastic process, microcrystals, debris and calcified collagenous bundles. Acids of sufficient concentration, by demineralizing many of these narrowing structures, apparently opened the tubules to the point of allowing the dye to penetrate. Interestingly, dyes have been found in vivo to readily penetrate active carious lesions but not arrested carious lesions (Miller and Marsh, 1962) further confirming that acid maintains patent tubules but if caries is arrested (i.e no further acid exposure) debris, sclerotic dentin, debris, etc. will occlude the tubules. In addition, Pashley et al. (1977) showed that the permeability of dentin to various substances is directly proportional to the molecular dimension of the substance and that size is more important than electrical charge in determining dentin permeability to the molecule.

Even though other acids (e.g. citric) are frequently utilized for demineralization studies, it was not surprising that lactic acid closely

mimiced in vivo caries, as it frequently has been implicated as a major contribution to human caries. (Geddes, 1975; Budz et al., 1988). Also, while the importance of recent work on absorption of weak acid anions to apatite was recognized and appreciated (Hoppenbrowers and Driessens, 1988; Budz et al., 1988), we did not attempt to quantitate or evaluate it in this particular model in this early developmental stage.

While the pH changes produced by lactic and citric acids were quantitatively similar, lactic acid produced the changes more gradually (Fig. 2 and 3) and did not result in crystal formation which, in turn, interfered with electron microscopic evaluation of the discs. These needle-like crystals were eventually hypothesized to be calcium citrate crystals, an insoluble salt resulting from acid interaction with the calcium in the dentin itself. When the acids were allowed to interact with different PBS concentrations directly or across various types of filters, no precipitate occurred nor did PBS on both sides of dentin produce crystals. Acetic, phosphoric and citric acid produced the crystals only when the dentin discs were present. The amount of crystals was perceptibly greater on the pulpal side of the discs and varied in proportion to the acid concentration. Lactic acid did not produce the crystals. It is possible that these crystals may approximate the "caries crystals" described by Newbern in 1978. Finally, in addition to the absence of crystal production, the pH drop produced with 6% lactic acid, which was within clinically relevant parameters, was calculated to have resulted from the passage of approximately 2.5 uL of the acid into 1 mL of buffer.

To more closely approximate the in vivo physiologic milieu and because parallel studies (Grower et al., 1989, 1990) were producing data which were

slightly confusing and difficult to interpret, it became apparent that in addressing the complex system of interactions involved in delivery of active drug through dentin, a model as physiologically precise as possible was required. As a consequence, alterations, in addition to the "decaying" (acid treating) of the dentin, were incorporated into the model.

Indomethacin is highly protein bound (90+%) (Honore and Brodersen, 1983; Yeh, 1985; Diana et al., 1989) and pulpal fluid contains protein similar to plasma (Haldi and Wynn, 1963; Pashley et al., 1982). Also dentinal tubules contain a fluid rich in protein (Pashley et al., 1983a) and the amount of plasma protein in the pulp increases in inflammation (Mjor, 1985) as a consequence of protein extravasation into extra-cellular fluid. To incorporate the effects of these factors into our model, the composition of our pulpal incubation buffer (PBS) was formulated to include 6.5% bovine serum albumin which approximates the total protein content of normal blood plasma (Scientific Tables, 1971). It was anticipated that this more physiologic solution might provide a mechanism (the serum albumin) for transport and stabilization of the poorly water soluble indomethacin, as well as a colloidal effect or a potential osmotic gradient to oppose the hydrophilic (H_2O attracting) properties of the delivery vehicles (Grower et al., 1989). Based on studies by Pashley et al., (1984, 1985), it was anticipated that the inclusion of the protein might actually decrease the movement of drug across the dentin and it was also anticipated that this would be more apparent in acid treated vs. unetched dentin. In addition, indomethacin is a very insoluble and acidic compound with a pK_a of 2 - 3. The pH of the pulpal fluid was, therefore, also anticipated to affect the amount of drug proportioning itself on either

side of the dentin discs (Brune and Graf, 1978) and as the pH in inflammatory tissue becomes progressively more acidic (Robbins et al., 1984; Fleury, 1990), the pulpal side pH in our model was reduced to 6.8 + to approximate inflamed pulpal tissue.

As initial results showed (Table 3), there was in the absence of protein a threefold increase in drug movement thru undecalcified dentin regardless of time or vehicle. This, in turn, was speculatively attributed to the lack of protein in the pulpal side buffer creating a non-physiologic state due, in varying degrees to: the ion exchange properties of the tubules; the osmotic effect and hydrophilic nature of the delivery vehicles; the poor water solubility of indomethacin in addition to its protein binding; or most probably some combination of these factors. Indeed, following addition of protein to the pulpal side buffer solution, there was a complete reversal in drug movement patterns with at least a doubling of movement then occurring thru decalcified versus undecalcified discs (Tables 5 and 6). In addition, the large increase in pupal drug concentration at 48 vs. 24 hours was no longer present and, in some cases, there was a statistically significant decrease at 48 hours [e.g., passage thru decalcified discs stabilized while movement thru undecalcified discs either stabilized (Table 5) or showed an increase (Table 6) in drug movement]. The basis for this apparent reverse movement of drug thru decalcified discs at pH 7.6, but not at pH 6.8, is at this point only speculative. As stated above, however, it may be attributable to the ion exchange properties of the dentinal tubules, the dissociation or ionization of the indomethacin molecule in the more acidic media, or adsorption of the ions to tubules. It may also be related to the acid pH in conjunction with the poor water solubility of indomethacin as well as its affinity for protein

and the hydrophilic nature of the delivery vehicles. Regardless, the purpose of this study was to determine if the physiologic alterations introduced into the model would affect drug movement, and not how. The specific mechanisms will require additional studies which are encouraged as comprehending the mechanisms will allow their manipulation to clinical advantage.

Finally, since decalcifying dentin with 6% lactic acid, as in caries, has an obvious effect (Tables 3,5,6), removing that variable thru the use of dialysis tubing enabled separate evaluation of the other 2 variables, pH and protein. The results shown in Table 6 readily demonstrated that both presence and absence of protein and the pH changes of \pm one-half unit clearly altered the movement of drug while the obvious differences between the various treatment modalities shown in Tables 3, 5, and 6 were not always statistically significant. This is primarily a consequence of the large standard error observed in some of the groups. This variation, in turn, appeared due in large part to the inherent biologic variation in human dentin discs which exhibit a degree of non uniformity whether used in the undecalcified or decalcified form.

In summary, decayed (acid treated) discs demonstrated different drug movement patterns compared to non-demineralized (unacid-etched) discs and the direction and degree of the changes, in turn, were found to be dependent on other variable parameters of the model system. For example, the addition of protein increases the movement of drug thru simulated decayed (acid treated) dentin; if the pH is inflammatory (6.8), drug movement stabilizes across decalcified discs between 24 and 48 hours, but continues to increase thru undecalcified discs; and if the "pulpal" pH is physiologic (7.6), there appears to be a reverse movement back across the dentin after 24 hours.

CONCLUSION

We have described an in vitro model designed to evaluate and measure the degree of drug movement thru "decayed (acid treated) dentin" into "inflamed pulpal" tissue. As the results indicate, each of the several individual factors incorporated into this model affected drug movement in different ways and to varying degrees. As each adds additionally to the creation of a model which more closely approximates the in vivo physiologic state, it is concluded that similar parameters be incorporated into in vitro models when studies are directed at predicting and optimizing movement of drug through carious dentin and into inflamed pulps in vivo.

"The views of the authors do not purport to reflect the views of the Department of the Army or the Department of Defense (Para. 4-3, AR 360-5)."

Materials disclaimer

Use of commercial products in this research does not reflect Government endorsement.

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TABLE 1

LABELED INDOMETHACIN COMPOUNDS TESTED FOR
PENETRATION THROUGH HUMAN DENTIN DISCS

DELIVERY VEHICLE	ISOTOPE	SPECIFIC RADIOACTIVITY: uCi/mg INDO
Diprobace Cream (a) (hydrophilic)	³ _H	2.4 uCi/mg
Inteban Gel® (b)	¹⁴ _C	0.24 uCi/mg
White Petrolatum (c) (hydrophobic)	¹⁴ _C	0.52 uCi/mg
Glycerin-Polyethylene Glycol CPD (c) (hydrophilic)	¹⁴ _C	0.52 uCi/mg
Polyethylene-Propylene glycol CPD (c) (hydrophilic)	¹⁴ _C	0.52 uCi/mg
Modified Hydrophylic Ointment (c)	¹⁴ _C	0.52 uCi/mg

- (a) [³H] Indomethacin labeled compounds were prepared by Amersham International by an exchange process and the product purified by HPLC.
- (b) Inteban Gel® was obtained from Sumitomo Chemical Co. 20 uCi of 2-¹⁴C Indomethacin obtained from Amersham International (SA = 31mCi/mMol) was added to 10 g of the gel to label the CPD.
- (c) 0.25 mL of DMSO was used as a solvent to dissolve 18 uCi of 2 - ¹⁴C Indomethacin (Amersham International, 31mCi/mMol) and 34.4 mg of unlabeled Indomethacin (Sigma Corp.) which was incorporated into 10 g of each delivery vehicle. Thorough incorporation of the cold and labeled Indomethacin was accomplished by gently warming each vehicle to just its melting point and adding the DMSO to the liquified vehicle and stirring thoroughly.

TABLE 2

PENTRATION OF 0.015% COOMASSIE BRILLIANT BLUE DYE
SOLUTIONS THROUGH DENTIN SLABS (a)

GROUP	ug DYE/mL OF BUFFER (b)	
	1 HOUR	24 HOURS
Non Acid Treated Dentin	0	0
Dentin Treated with 6% Lactic Acid for 48 Hours	0.48 \pm 0.29	0.93 \pm 0.36

(a) Dentin discs were inserted into the chambers used as the model pulp system. One (1) mL of 0.01 M PBS was placed on the pulpal side of the disc and 0.5 mL of 0.015% coomassie brilliant blue dissolved in 0.01 M PBS was placed the occlusal side of the disc. The chambers were then incubated at 37°C. At 1 hour and 24 hours, 0.5 mL samples of buffer were taken from the pulpal side for measurement of the dye penetration through the dentin based on the measurement of the absorbance of the samples at 550 nanometers with a Guilford Spectrophotometer.

(b) Mean \pm SEM (n=4)

TABLE 3

PENETRATION OF [^{14}C] AND [^3H] LABELED
INDOMETHACIN THROUGH HUMAN DENTIN DISCS
NO SERUM ALBUMIN IN INCUBATION BUFFER

GROUP (a)	DISC TREATMENT	INDOMETHACIN PENETRATION (b)	
		24 HR	48 HR
		ug/mL	
<hr/>			
	¹⁴ C Labeled		
Modified Hydrophilic Ointment (n=4)	Decalcified	3.7 ± 0.6	23.5 ± 8.1
	Undecalcified	7.2 ± 1.6*	25.6 ± 4.7
White Petrolatum (n=3)	Decalcified	0.9 ± 0.4	1.1 ± 0.3
	Undecalcified	2.6 ± 0.6*	2.3 ± 0.3*
<hr/>			
	³ H Labeled		
Diprobace Cream (n=3)	Decalcified	3.2 ± 0.5	9.8 ± 2.4
	Undecalcified	9.1 ± 1.7*	14.7 ± 5.6
<hr/>			

(a) 400 mg samples of test compounds were put in the occlusal section of each chamber and incubated at 37°C against 1 mL of plain pH 7.6 (0.01 M) PBS buffer on the pulpal side of the dentin disc. After 24 HRS, the buffer was removed and new buffer was placed in the chamber for an additional 24 HRS of incubation.

(b) Mean \pm SEM.

* t Test $p < 0.05$ Decalcified versus Undecalcified Discs

TABLE 4

DIFFUSION OF ^{14}C LABELED INDOMETHACIN
IN POLYETHYLENE-PROPYLENE GLYCOL VEHICLE
THROUGH DIALYSIS TUBING

SAMPLE SIZE	BUFFER SYSTEM (a)	INDOMETHACIN DIFFUSION ug/mL (b)	
		24 HR	48 HR
6	pH 7.6 PBS +6.5% SER ALB	28.8 \pm 8.2	21.0 \pm 5.6
3	pH 7.6 PBS NO SER ALB	2.25 \pm 0.4	3.6 \pm 0.56
3	pH 6.8 PBS + 6.5%SER ALB	9.8 \pm 3.3	4.5 \pm 2.6
3	pH 6.8 PBS NO SER ALB	3.0 \pm 1.5	3.0 \pm 0.9

(a) 200 mg samples of the test compound were placed in the occlusal section of each chamber, with a single thickness of dialysis tubing separating it from the pulpal chamber containing 1 mL of buffer, and the chambers were incubated at 37°C. At 24 HRS, the pulpal buffer was removed and new buffer was added for an additional 24 HRS incubation.

(b) MEAN \pm SEM

ANOVA - WITHIN GROUP COMPARISON	24 HR	p = 0.013
	48 HR	p = 0.0005

TABLE 5

¹⁴C LABELED INDOMETHACIN IN POLYETHYLENE-PROPYLENE GLYCOL VEHICLE
PENETRATION THROUGH DENTIN DISCS pH 7.6 VS 6.8

GROUP (a)	¹⁴ C INDOMETHACIN PENETRATION ug/mL (b)	
	24 HR	48 HR
Decal pH 7.6 + 6.5% Ser Alb n = 4	8.21 \pm 2.39	7.69 \pm 2.59
Decal pH 6.8 + 6.5% Ser Alb n = 4	6.94 \pm 0.94*	8.34 \pm 0.45**
Un Decal pH 6.8 + 6.5% Ser Alb n = 3	3.59 \pm 1.4	3.70 \pm 1.09

(a) 200 mg samples of the test compound were placed in the occlusal section of each chamber, with a dentin disc separating it from the pulpal chamber containing 1 mL of buffer, and the chambers were incubated at 37°C. At 24 HRS, the pulpal buffer was removed and new buffer was added for an additional 24 HRS incubation.

(b) Mean \pm SEM

* p<0.05 Decal pH 6.8 vs. Undecal pH 6.8

** p<0.005 Decal pH 6.8 vs. Undecal pH 6.8

TABLE 6

EFFECT OF pH OF ELUTION BUFFER
ON PENETRATION OF [14 C] LABELED INDOMETHACIN
THROUGH HUMAN DENTIN

TREATMENT OF DENTIN DISCS	pH OF BUFFER	VEHICLE (a)	INDOMETHACIN PENETRATION (b) ug/mL	
			<u>24 HR</u>	<u>48 HR</u>
		<u>INTEBAN GEL</u>		
Decalcified	7.6		21.2 \pm 3.8	15.5 \pm 0.9
Decalcified	6.8		22.1 \pm 7.9	22.2 \pm 3.4
Undecalcified	6.8		10.0 \pm 1.8	14.0 \pm 3.1
		<u>GLYCERIN- POLYETHYLENE GLYCOL CPD</u>		
Decalcified	7.6		7.9 \pm 1.3	5.9 \pm 1.2
Decalcified	6.8		6.3 \pm 3.0	6.3 \pm 2.0
Undecalcified	6.8		1.2 \pm 0.2	2.4 \pm 0.2

(a) 200 mg of each compound tested were placed in the chambers (which were incubated at 37°C) with a human dentin disc separating it from 1 mL of elution buffer at the indicated pH containing 6.5% serum albumin. After 24 HRS, the buffer in the pulpal chambers was removed and 1 mL of new buffer was added and incubated for another 24 HRS.

(b) Mean \pm STD error of the mean. 4 samples in each group.

LEGENDS

- Fig. 1 Schematic drawing of the delrin plastic chamber used as the tooth model. The dentin discs were sealed between the occlusal and pulpal chambers by means of the "O" rings shown.
- Fig. 2 pH changes in the pulpal buffer after treatment of dentin discs at 25°C with various acids. Dentin discs were incubated with 1 mL of 0.002 M PBS at pH 7.6 in the pulpal buffer reservoir at the start of the incubation period.
- Fig. 3 Effects of buffer concentration and incubation temperature on pH changes in the pulpal buffer after exposure of dentin discs to 6% lactic acid. Dentin discs were incubated with 1 mL of PBS in the pulpal reservoir at pH 7.6 with the molarity indicated at the start of the incubation period.
- Fig. 4 Scanning electron microscopic views of the pulpal and occlusal aspect of human dentin discs.
- a = view of the pulpal side of the dentin from natural caries (4000X).
 - b = view of the pulpal side of dentin decalcified with 6% lactic acid for 48 hours in the model system (4000X).
 - c = view of the outer (occlusal) side of dentin from natural caries (4000X).
 - d = view of the outer (occlusal) side of dentin decalcified with 6% lactic acid for 48 hours in the model system (4000X).
- Fig. 5 SEM view outer (occlusal) side of untreated dentin discs (2000X) and (4000X).

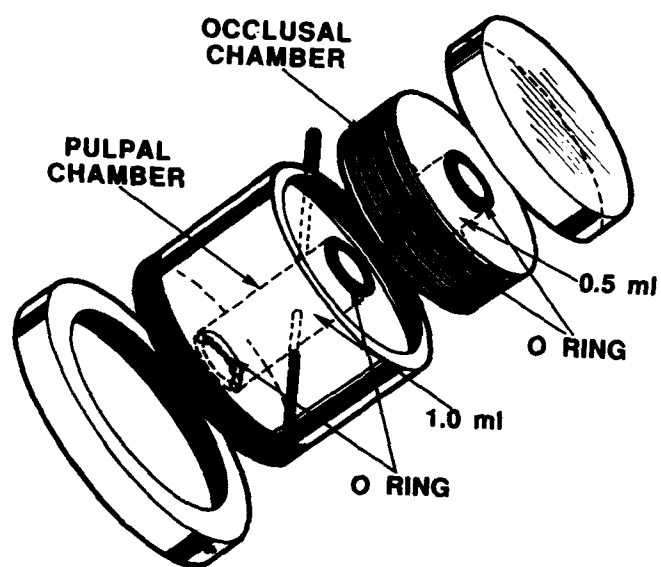


Fig. 1 Schematic drawing of the delrin plastic chamber used as the tooth model. The dentin discs were sealed between the occlusal and pulpal chambers by means of the "O" rings shown.

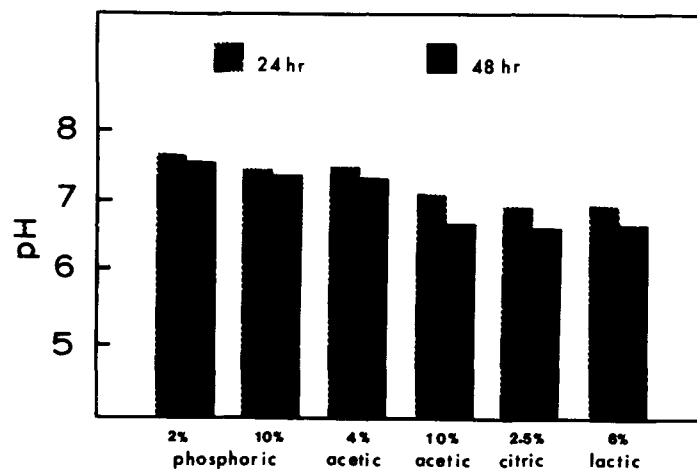


Fig. 2 pH changes in the pulpal buffer after treatment of dentin discs at 25°C with various acids. Dentin discs were incubated with 1 mL of 0.002 M PBS at pH 7.6 in the pulpal buffer reservoir at the start of the incubation period.

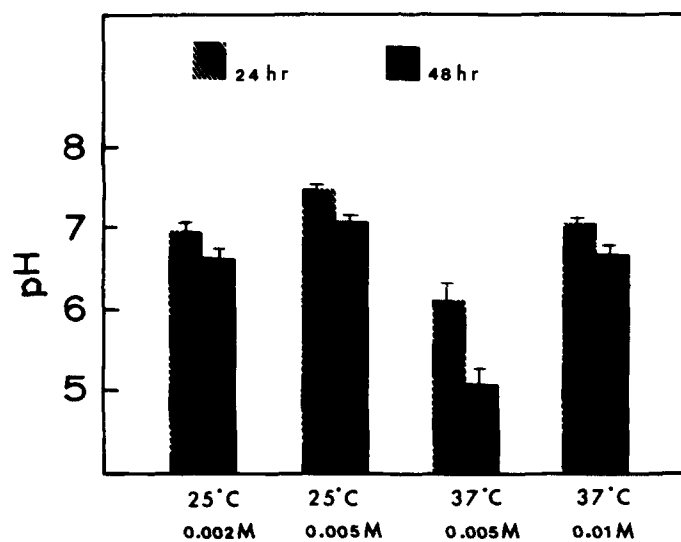


Fig. 3 Effects of buffer concentration and incubation temperature on pH changes in the pulpal buffer after exposure of dentin discs to 6% lactic acid. Dentin discs were incubated with 1 mL of PBS in the pulpal reservoir at pH 7.6 with the molarity indicated at the start of the incubation period.

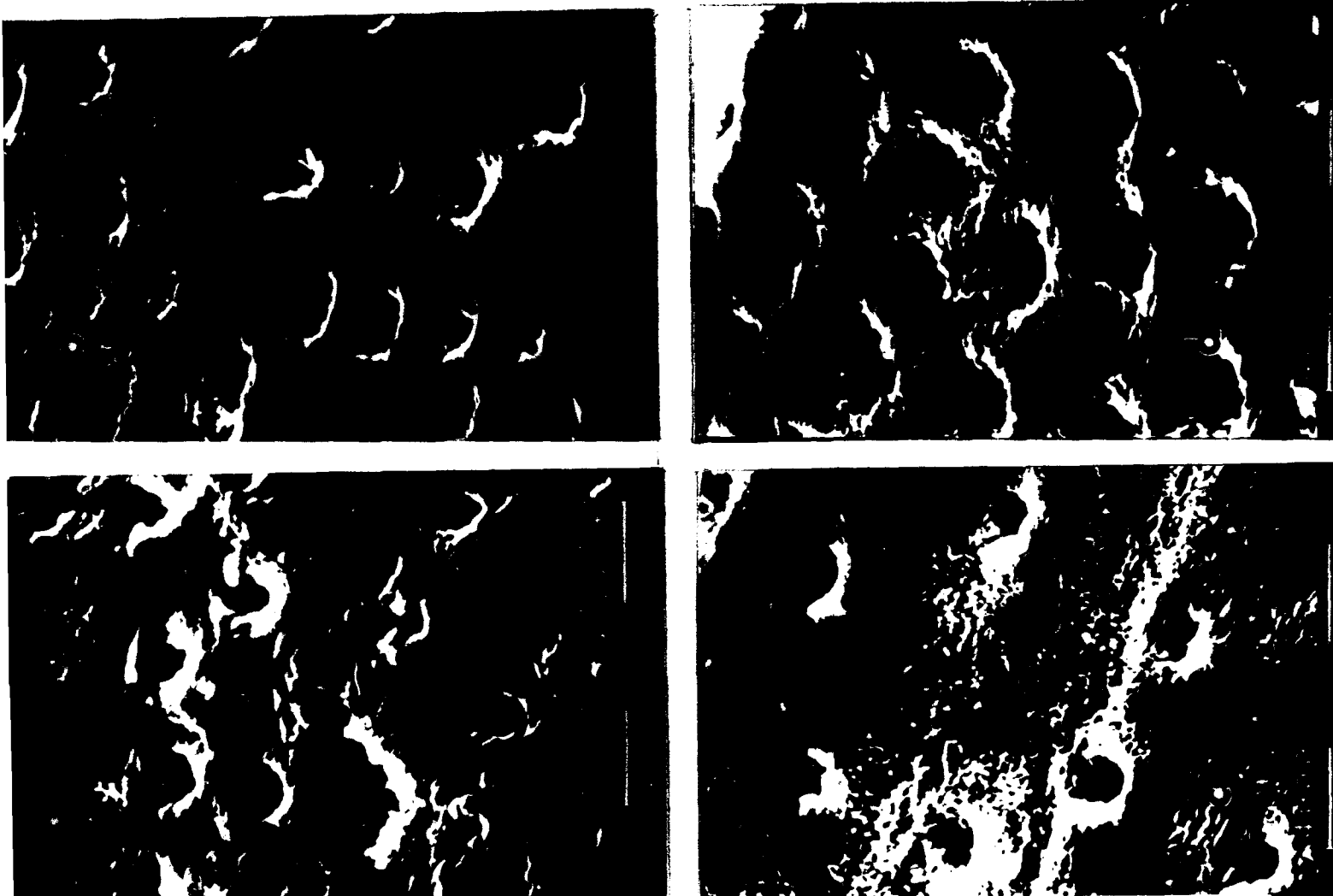


Fig 4 Scanning electron microscopic views of the pulpal and occlusal aspect of human dentin discs.

- a - view of the pulpal side of the dentin from natural caries (4000X).
- b - view of the pulpal side of dentin decalcified with 6% lactic acid for 48 hours in the model system (4000X).
- c - view of the outer (occlusal) side of dentin from natural caries (4000X).
- d - view of the outer (occlusal) side of dentin decalcified with 6% lactic acid for 48 hours in the model system (4000X).

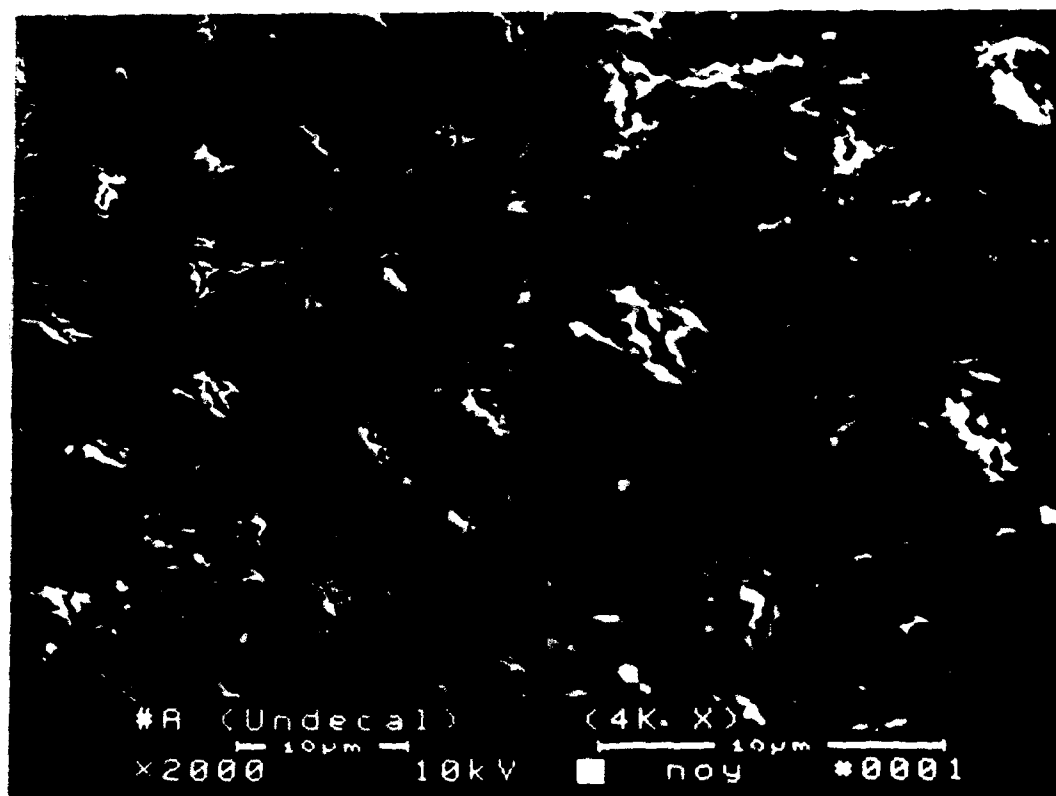


Fig. 5 SEM view outer (occlusal side of untreated dentin discs (2000X) and (4000X).